Cholesterol and saturated fatty acids synergistically promote the malignant progression of prostate cancer

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Abstract

The excessive accumulation of saturated fatty acids and cholesterol have been linked to prostate cancer (Pca). Here, we found that lipoproteins, apolipoproteins, triglycerides and free fatty acids are significantly higher in the peripheral blood of prostate cancer patients than in non-cancer patients. Furthermore, the expression of ACC1, FASN and HMGCR is significantly higher in prostate cancer tissues than that in non-cancer tissues, and positively correlated with the gleason score. Using genetically engineered mouse models, we found that in a mouse model of high grade prostatic intraneoplasia (HGPIN), a combination of fatty acid synthase (FASN) overexpression and cholesterol efflux pump (Abca1) knockout resulted in the progression of prostatic intraneoplasia (PIN) to invasive PCa with 100% penetrance, as well as an increase in prostate cancer stem cell (PCSC)population, accompanied by activation of PGE$_2$ and TGF-β signaling pathway. Our study suggests that the steady rise in prostate cancer incidence and mortality among Chinese population during the last several decades may be attribute to a combinational effect of fatty acid and cholesterol, and reduction in dietary fat and cholesterol intake could slow down the progression from occult lesions to prostate cancer.

Introduction

Prostate cancer ranks as the most commonly diagnosed malignancy and the second leading cause of cancer death in American men. It is estimated that 1 in 6 men will be diagnosed with prostate cancer during their lifetime (1).

Although the incidence of prostate cancer in Western countries is significantly higher than that in Asian countries, the incidence of asymptomatic and occult prostate cancer accompanied by genetic mutations is almost the same worldwide. This contradiction was discovered decades ago and has not yet been resolved.

Although the threat of prostate cancer in China is relatively lower than that in the Western developed countries, the incidence and fatality rate of prostate cancer is rising continuously, and the average annual growth rate of deaths in the past ten years is 8.44% (63,64, in Chinese). Therefore, explore the underlying reasons and new preclinical prevention or treatment measures is urgently needed.

A potential link between cholesterol and cancer was suggested almost a century ago (2,3). The cholesterol levels are increased in benign hyperplastic prostate compared to normal prostate (4) and in prostate cancer bone metastases compared to bone metastases of other cancers (5). Preclinical studies show that cholesterol promotes prostate cancer progression (6-8) and a high level of circulating cholesterol has been associated with aggressive forms of the disease (9–12), though this conclusion is not consistent (13). Stronger evidence comes from multiple studies on cholesterol-lowering drugs (statins) and prostate cancer risk. Four independent large epidemiological studies find a protective effect of statins against advanced prostate cancer (14–17). An

editorial (18) conveyed the excitement of these findings at the time, where consistent results across studies are a rarity.

Several lines of evidence suggest a positive association between dietary fat intake and prostate cancer risk. Ecological studies report that countries with higher per capita consumption of fat have higher rate of prostate cancer mortality (19,20). Epidemiological studies show that both higher fat intake and higher plasma palmitoleic, palmitic and alpha-linolenic acid levels are associated with increased risk of prostate cancer or prostate cancer progression (21–23). Consistent with this concept, high body mass index, body fat, and obesity are all positively correlated with prostate cancer mortality (24–26). In addition, animal studies using xenograft or transgenic mouse prostate tumor models show that a high-fat diet promotes prostate cancer progression (27–29).

However, the relationship between saturated fatty acids or cholesterol with prostate cancer is controversial (7,30–33). Here, we identified saturated fatty acids and cholesterol synergistically promote the proliferation of prostate cancer stem cells, and lead to the deterioration of prostate cancer. Our results provide a possible explanation for the discrepancy in prostate cancer occurance among the Asian and Western populations as well as the recent rise in incidence and mortality in China.

Results

Prostate cancer has altered lipid metabolism

In China, prostate cancer has become the only male malignant tumor with a significant increase in both morbidity and mortality in the last two decades (34,35). To identify possible causes, we designed a hospital-based epidemiological survey. We selected 1184 representative cases between 2010 and 2017 from the patients at the Cancer Registry of Wuxi CDC and 507 age-matched control derived from an unbiased sampling in Wuxi population (Table S1). By analyzing 58 biochemical and cellular markers, we found that levels of 8 lipid related markers as well as the prostate cancer specific antigen (PSA) and age were significantly associated with cancer risk (Fig. 1A and Tables S2,S3). Next, we collected 150 specimens of prostate cancer and 50 non-cancerous prostate tissues, and evaluated the expression of lipid metabolic markers by immunohistochemistry. Our results showed that levels of acetyl-CoA carboxylase (ACC1), fatty acid synthase (FASN) and HMG-CoA reductase (HMGCR) were significantly higher in prostate cancer than that in non-cancer tissues (Fig. 1B,C). Moreover, the expression of FASN, HMGCR and ACC1 in prostate cancer tissue was positively correlated with Gleason score (Fig. 1D,E). To further corroborate this result, we used prostate cancer tissue array (100 prostate cancer specimens and 50 corresponding adjacent tissues) and consistent results were obtained (Fig. 1F,G).

Prostate-specific deletion of Abca1 has no effect on mouse prostate development

FASN is the enzyme responsible for *de novo* lipid synthesis, whereas ACC1 and HMGCR are a rate-limiting enzyme in fatty acid and cholesterol synthesis, respectively. To assess the effect of cholesterol on prostate cancer, we knocked out *Abca1*, a cellular cholesterol efflux protein, in the prostate. Prostate-specific *Abca1* knockout mice ($Abca1^{-/-}$) showed increased cholesterol levels in the prostate but not in the liver or plasma (Fig. S1A). However, neither prostate weight (Fig. S1B) nor histology (Fig. S1C) was affected as a result of increased cholesterol in the prostate.

Prostate-specific overexpression of FASN does not significantly affect mouse prostate development

To determine the effect of excess fatty acids on the prostate, we generated prostate-specific *FASN* overexpression mice ($FASN^T$). Fatty acid methyl ester analysis revealed a significant increase in palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), as well as total fatty acids in the transgenics compared to the wild type mice (Fig. S2A). Despite the significant difference in fatty acid levels, no change in prostate weight was observed (Fig. S2B). Although a previous report showed that $FASN^T$ mice developed PIN as early as 3 months, nearly all $FASN^T$ mice in our study showed normal pathology for up to 12 months in all three lobes of the prostate; only 16% of mice showed some signs of benign hyperplasia (Fig. S2C).

Prostate-specific Abca1 deletion and FASN overexpression induces prostate hyperplasia in mice

Since increases in fatty acids or cholesterol alone had little effect on mouse prostate, we crossed $Abca1^{+/-}$ with $FASN^T$ mice to examine the combined effect of both cholesterol and fatty acid on the prostate. Compared to $Abca1^{+/+}$; $FASN^T$, $Abca1^{-/-}$; $FASN^T$ mice showed a significant increase in cholesterol in the prostate (Fig. 2A), had increased prostate weight and pathological development (Fig. 2B). 50 % of $Abca1^{-/-}$; $FASN^T$ mice developed prostatic hyperplasia by 6 months, which increased to 100% by 12 months (Fig. 2B and C). In comparison, 0% of $Abca1^{-/-}$ (Fig. 2B) and 16% of $FASN^T$ mice developed hyperplasia.

Prostate-specific Abca1 deletion and FASN overexpression promote high grade prostatic intraneoplasia in Pten$^{+/-}$ mice

Microscopic cancer lesions often harbor genetic mutations in oncogenes or tumor suppressor genes. Activation of phosphoinositide 3-Kinase (PI3K) pathway is observed in nearly all advanced prostate cancers. The loss of phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is the most common signaling aberration found in prostate cancers. Reports have identified that up to 40% of prostate cancer patients have a heterozygous deletion of *PTEN* (36). We sought to determine whether the combination of cholesterol and fatty acids can affect prostate cancer progression in *Pten* heterozygous background. $Abca1^{-/-}$; $FASN^T$ mice were crossed with the *Pten* knockout prostate cancer mouse model. We first determined whether high cholesterol and fatty acid levels were sufficient to accelerate tumorigenesis in *Pten* heterozygous mice. At 6 months of age, 25% of $Pten^{+/-}$ mice developed hyperplasia. $Pten^{+/-}$; $Abca1^{-/-}$ mice showed the same pattern. 50 % of $Pten^{+/-}$; $FASN^T$ mice had lesions with 17% hyperplasia and 33% high-grade prostatic intraneoplasia (HGPIN). All $Pten^{+/-}$; $Abca1^{-/-}$; $FASN^T$ mice developed lesions with 50% hyperplasia and 50% HGPIN (Fig. 3A). At 12 months of age, all groups of mice developed HGPIN (Fig. 3A and B) with similar prostate weight (Fig. 3A).

Prostate-specific Abca1 deletion and FASN overexpression transform high grade prostatic intraneoplasia into invasive carcinoma in Pten$^{-/-}$ mice

Next, we determined whether the combination of cholesterol and fatty acids accelerated tumorigenesis in *Pten* homozygous background. Data showed higher cholesterol levels in $Pten^{-/-}$; $Abca1^{-/-}$ mice compared to $Pten^{-/-}$; $Abca1^{+/+}$ mice (Fig. 4A). Because $FASN^T$ mice express luciferase, *in vivo* imaging results showed that luminescence was much greater in $Pten^{-/-}$; $Abca1^{-/-}$; $FASN^T$ mice compared to either $FASN^T$ alone or $Pten^{-/-}$; $FASN^T$ mice, suggesting combination of $Abca1^{-/-}$ and $FASN^T$ significantly increased tumor burden (Fig. 4B). All genotypes developed HGPIN with 100% penetrance at 6 months (Fig. 4C). At 12 months, $Pten^{-/-}$ and $Pten^{-/-}$; $Abca1^{-/-}$ mice still showed only HGPIN lesions, whereas 33% of prostates from $Pten^{-/-}$; $FASN^T$ mice and 100% of prostates from $Pten^{-/-}$; $Abca1^{-/-}$; $FASN^T$ mice had progressed to invasive carcinoma (Fig. 4C, D). $Pten^{-/-}$; $Abca1^{-/-}$; $FASN^T$ mice had a significantly higher prostate weight compared
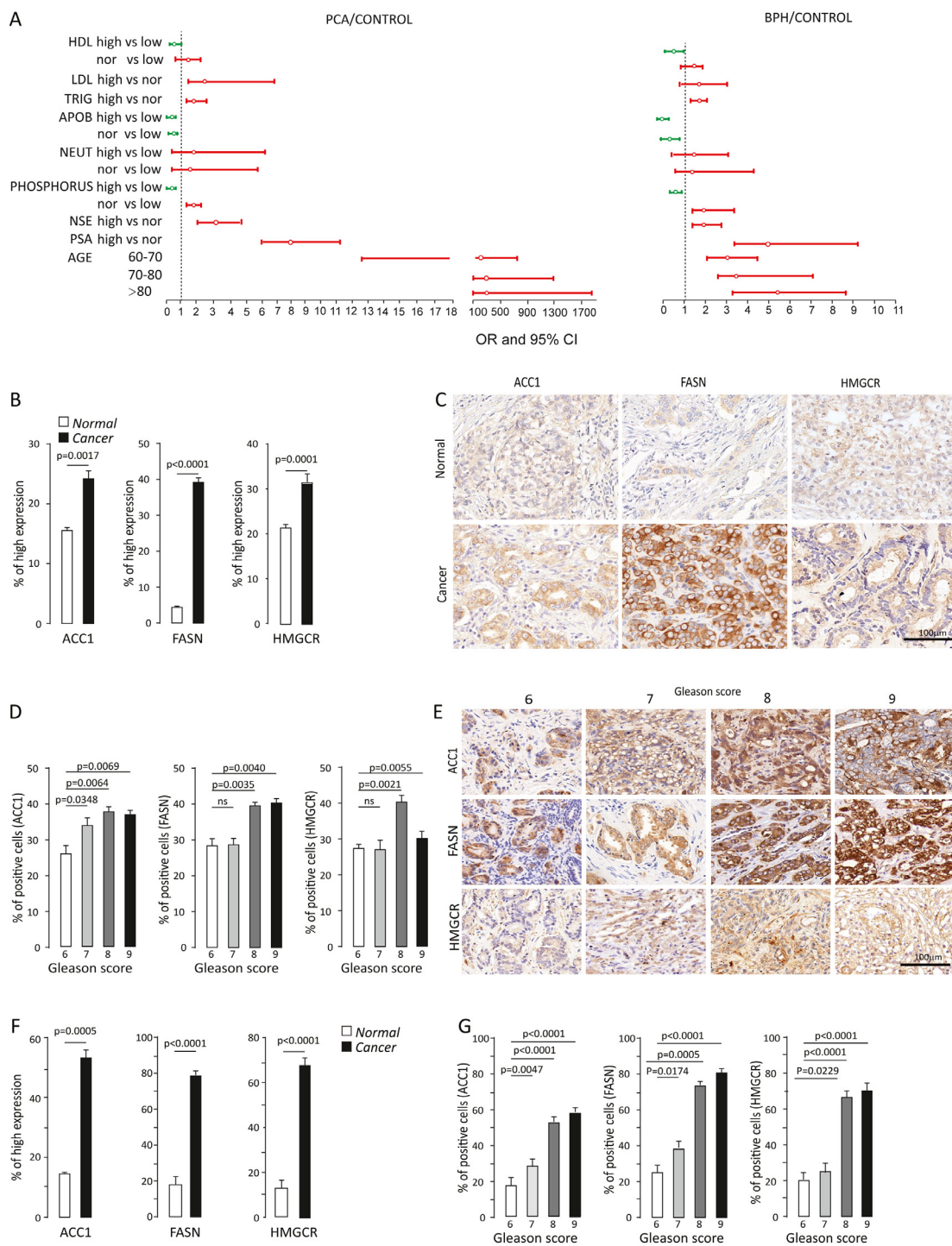
Prostate cancer has altered lipid metabolism

Fig. 1. Prostate cancer has altered lipid metabolism.
(A) Correlation analysis between prostate cancer and lipid metabolism. A preliminary retrospective study on the biochemical indicators of patients with prostate cancer and benign prostatic hyperplasia. HDL: high-density lipoprotein; LDL: low-density lipoprotein; TRIG: triglycerides; APOB: apolipoprotein B; NEUT: neutrophils; NSE: neuron-specific enolase, AGE: years Age; PSA: prostate cancer specific antigen. (B-C). Statistical analysis of IHC for prostate cancer and adjacent tissues: The expression of FASN, HMGCR and ACC1 in prostate cancer tissue is significantly higher than that in the corresponding adjacent tissues. (D-E). Correlation analysis between Gleason score and related indexes of lipid metabolism. (F-G). Correlation analysis between Gleason score and related indexes of lipid metabolism in prostate cancer tissue array.
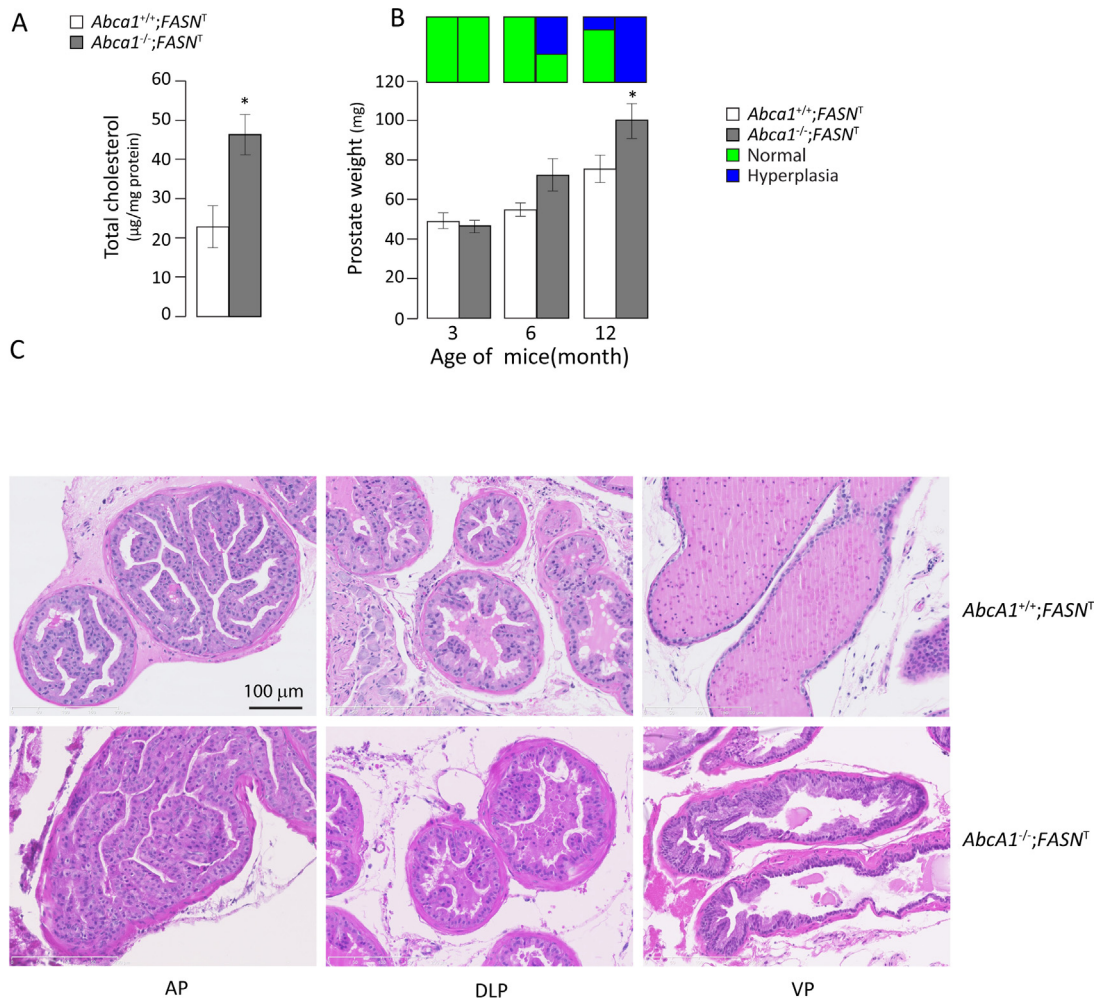
Prostate-specific *Abca1* deletion and *FASN* overexpression induces prostate hyperplasia in mice
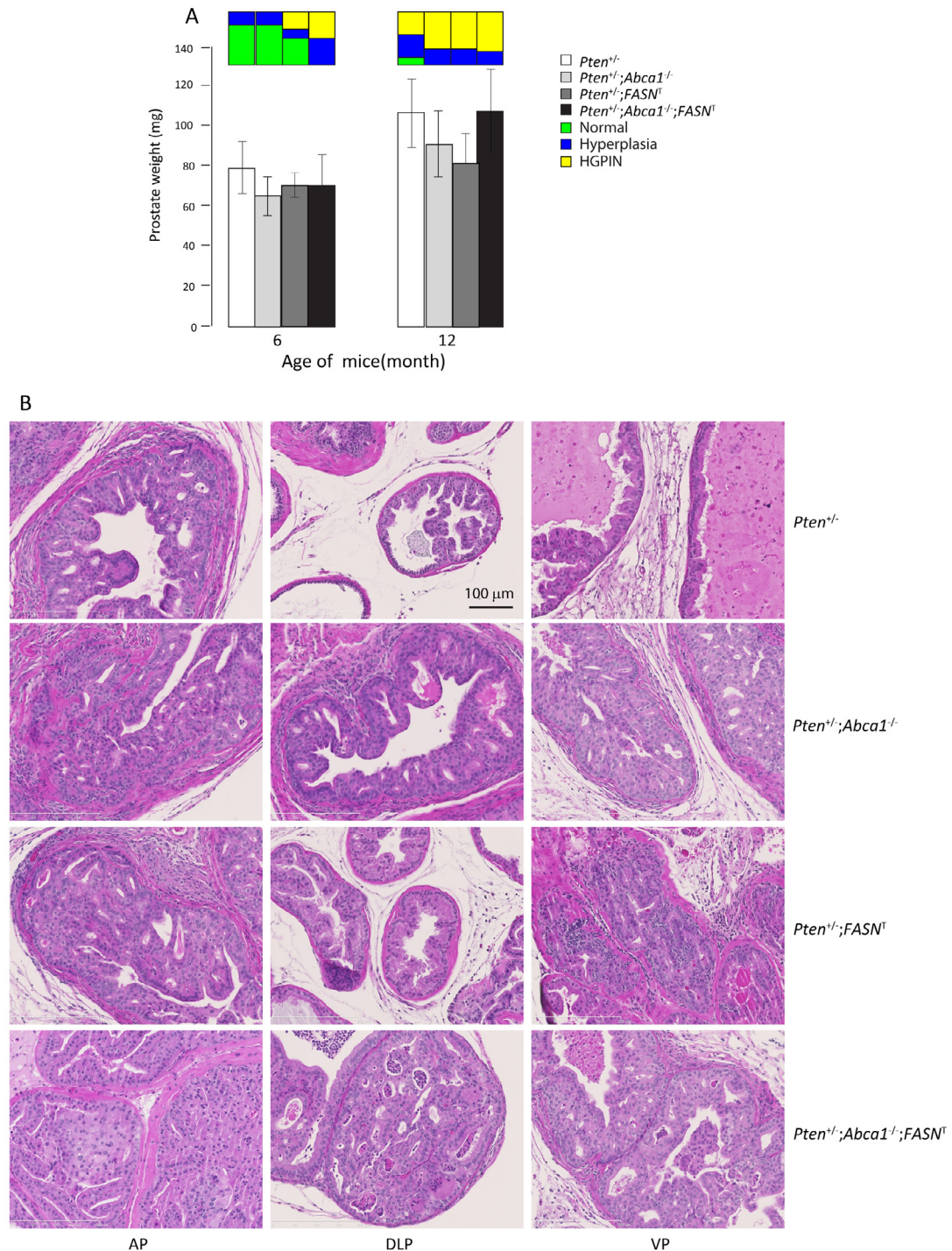
Fig. 2. Prostate-specific *Abca1* deletion and *FASN* overexpression induces prostate hyperplasia in mice

(A). $Abca1^{-/-}$; $FASN^T$ had increased prostate cholesterol level compared to $Abca1^{+/+}$; $FASN^T$ mice ($p<0.05$, Student t-test). (B). $Abca1^{-/-}$; $FASN^T$ mice grew larger prostates compared to $Abca1^{+/+}$; $FASN^T$ mice at 12 months of age ($p < 0.05$, Student t test). * indicates a statistically significant difference. $Abca1^{-/-}$; $FASN^T$ mice developed significantly higher percentage of hyperplasia ($p < 0.005$, χ^2 test). (C). Representative histological images of prostates from $Abca1^{+/+}$; $FASN^T$ and $Abca1^{-/-}$; $FASN^T$ mice at the age of 12 months. A cohort of 132 mice was used.

to $Pten^{-/-}$ mice at 3 months, and compared to $Pten^{-/-}$, $Pten^{-/-}$; $Abca1^{-/-}$, $Pten^{-/-}$; $FASN^T$ mice at 12 months (Fig. 4C). These results indicate that concomitant increase in cholesterol and fatty acids may aggressively accelerate disease progression.

Prostate-specific Abca1 *deletion and* FASN *overexpression up-regulates* PGE_2 / TGF-β *pathways in* $Pten^{-/-}$ *prostate cancer*

To evaluate the effects of $FASN^T$ and $Abca1^{-/-}$ on signaling pathways, we determined RNA expression in $Pten^{-/-}$ (named P), $Pten^{-/-}$; $Abca1^{-/-}$ (named PA), $Pten^{-/-}$; $FASN^T$ (named PF), and $Pten^{-/-}$; $Abca1^{-/-}$; $FASN^T$ (named PAF) prostate tissues by microarray(37). Our results showed that 2582 genes or transcripts were differentially expressed between P and PA,

2182 between P and PF, 3190 between P and PAF, and 1552 common among PA, PF, PAF (Fig. 5A). Pathway analysis suggested that the prostaglandin E_2 (PGE_2) and transforming growth factor β (TGF-β) pathway-associated genes were significantly altered in PAF (Fig. 5B). Up-regulation of very low-density lipoprotein receptor (*Vldlr*), LDL receptor related protein 1 (*Lrp1*) and 12 (*Lrp12*), LDL receptor related protein associated protein 1 (*Lrpap1*) can increase cell uptake of lipid such as linoleic acid (LA). LA is converted by fatty acid desaturase 1 (*Fads1*), 2 (*Fads2*) and 3 (*Fads3*) to arachidonic acid (AA) which is then metabolized by prostaglandin-endoperoxide synthase 1 (*Ptgs1*, aka *Cox1*) and 2 (*Ptgs2*, aka *Cox2*) into prostaglandins. PGE_2 binds to prostaglandin E receptor 3 (*Ptger3*) to exert its function. Down-regulation of prostaglandin reductase 2 (*Ptgr2*) and 15-hydroxyprostaglandin dehydrogenase (*Hpgd*), two major PGE_2 inactivating enzymes, helps to stabilize PGE_2 level.

Prostate-specific *Abca1* deletion and *FASN* overexpression promote high grade prostatic intraneoplasia in

Pten+/- mice

Fig. 3. Prostate-specific *Abca1* deletion and *FASN* overexpression promote high grade prostatic intraneoplasia in $Pten^{+/-}$ mice
(A). Prostate lesion development in different stages. (B). Representative histological pictures of prostates from $Pten^{+/-}$ mice at the age of 12 months. The anterior (AP), dorsolateral (DLP), and ventral (VP) prostatic lobes showed hyperplasia or PIN lesions. A cohort of 65 mice was used.
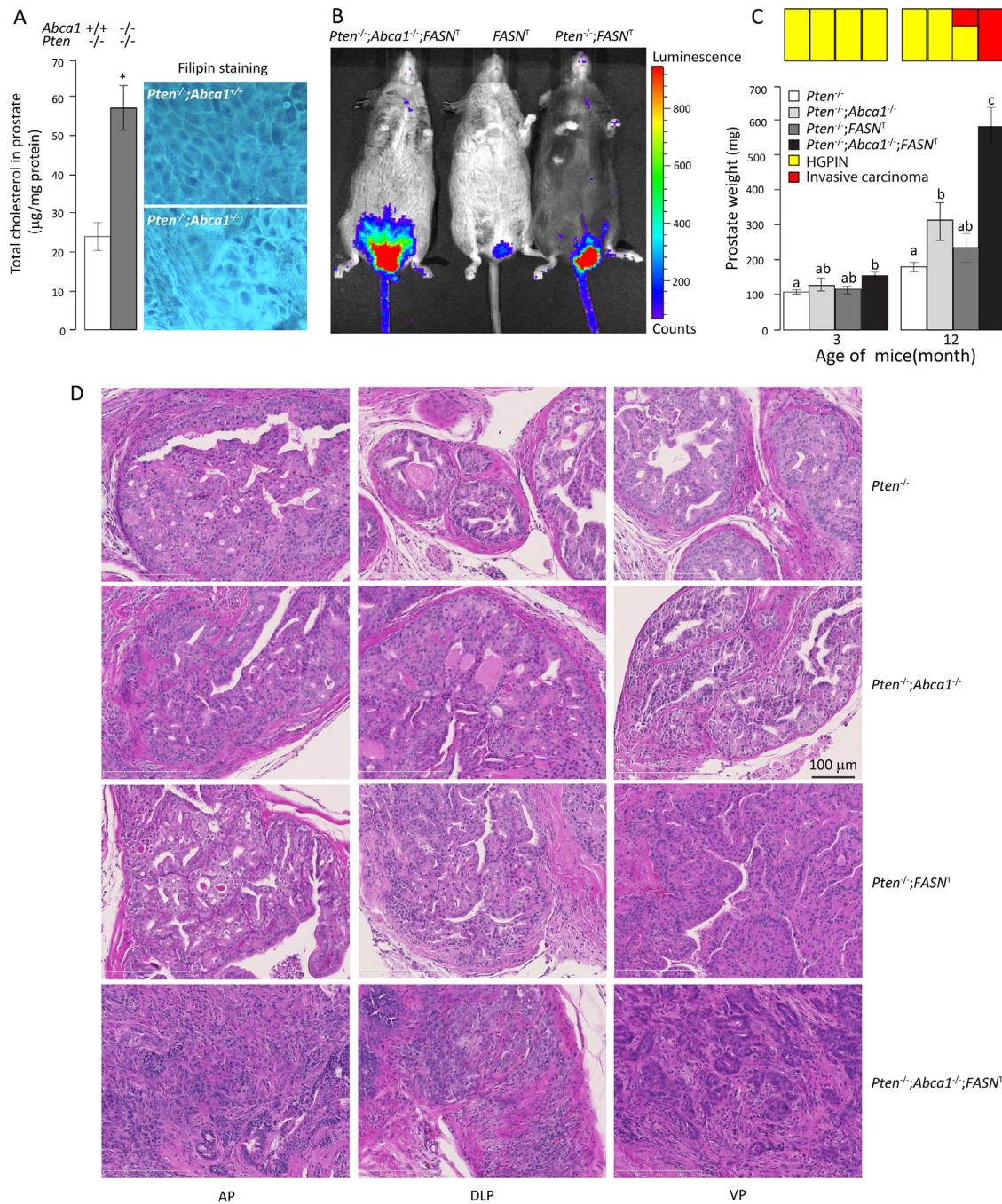
Abca1 deletion and *FASN* overexpression transforms HGPIN into invasive carcinoma in *Pten⁻/⁻* mice

Fig. 4. Prostate-specific *Abca1* deletion and *FASN* overexpression transform high grade prostatic intraneoplasia into invasive carcinoma in $Pten^{-/-}$ mice (A). Cholesterol levels were significantly increased in prostatic tumors from $Pten^{-/-}$; $Abca1^{-/-}$ compared to $Pten^{-/-}$; $Abca1^{+/+}$ mice ($p < 0.001$, Student t-test). Filipin staining demonstrated increased intensity of cholesterol signal in prostate tumors of $Pten^{-/-}$; $Abca1^{-/-}$ mice. (B). Transgene overexpression was demonstrated by the increased luciferase activity. Luciferase activity correlated with the prostate size. (C) Prostate changes in different genotypes of mice at different stages. Abbreviation used: N, normal; H, hyperplasia; P, PIN; INV, invasive prostate cancer. (D). Representative prostate tumor histology of $Pten^{-/-}$ mice at 12 months of age. Prostates showed PIN lesions in anterior (AP), dorsolateral (DLP), and ventral (VP) prostatic lobes. Invasive prostate cancers were seen in all lobes from $Pten^{-/-}$; $Abca1^{-/-}$; $FASN^{T}$ mice. A cohort of 75 mice was used in this study.
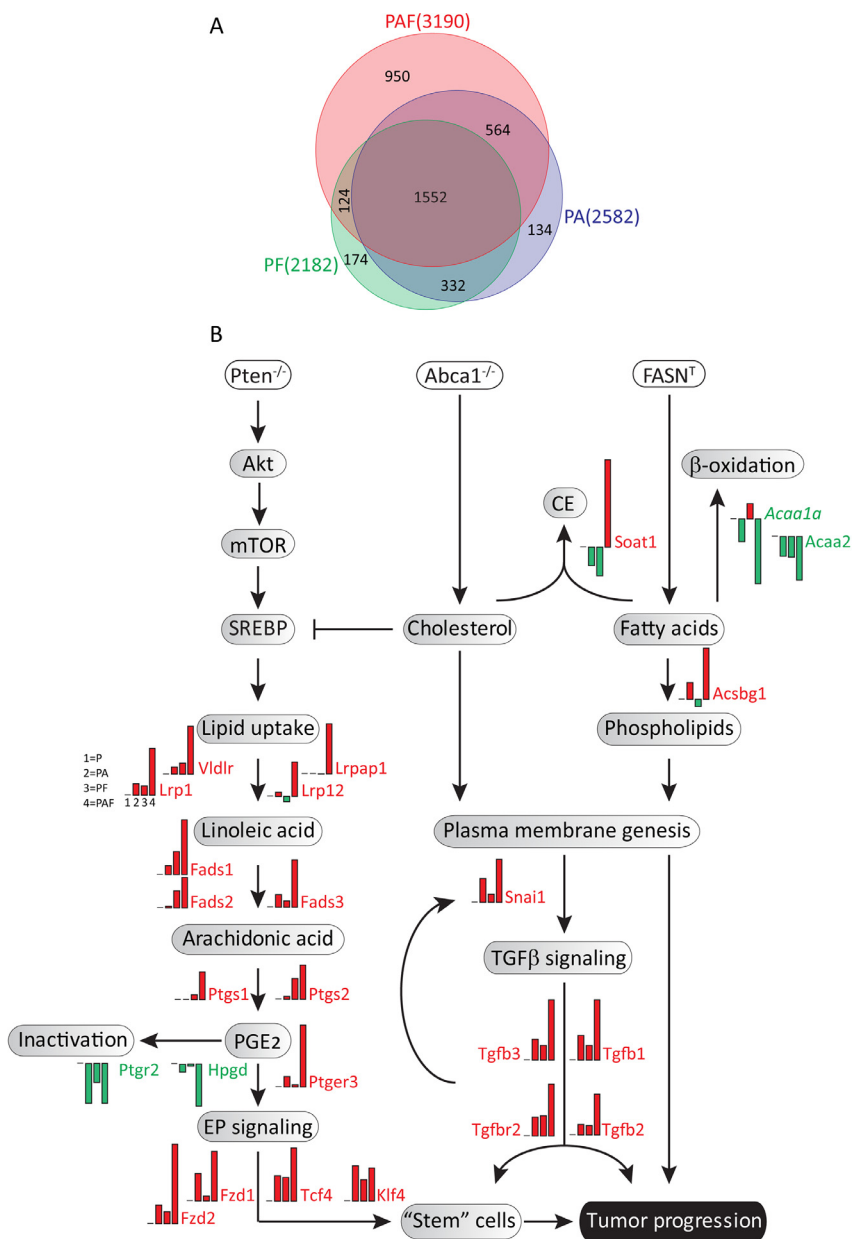
Prostate-specific *Abca1* deletion and *FASN* overexpression up-regulates PGE$_2$/ TGF-β pathways in *Pten*$^{-/-}$ prostate cancer

Fig. 5. Prostate-specific *Abca1* deletion and *FASN* overexpression up-regulates PGE$_2$ / TGF-β pathways in *Pten*$^{-/-}$ prostate cancer
(A). 1, 2, 3, and 4 in Lrp1 bar graph represent $P^{-/-}$, $P^{-/-}$; $A^{-/-}$, $P^{-/-}$; F^T and $P^{-/-}$; $A^{-/-}$; F^T, respectively. All bar graphs follow the the same order. Expression level in $P^{-/-}$ was used as the reference. Red bars indicate up-regulation, green bars down-regulation. Acaa1a: acetyl-CoA acyltransferase 1, Acsbg1: acyl-CoA synthetase bubblegum family member 1, Fads: fatty acid desaturase, Fzd: frizzled class receptor, Hgpd: hydroxyprostaglandin dehydrogenase 15, Klf4: Kruppel-like factor 4, Lrp: LDL receptor related protein, Lrpap1: LDL receptor related protein associated protein 1, Ptger3: prostaglandin E receptor 3 (EP3), Ptgs: prostaglandin-endoperoxide synthase (Cox), Snail1: snail family zinc finger 1, Soat1: sterol O-acyltransferase 1, Tcf4: transcription factor 4, Tgfb: Transforming growth factor beta, Tgfbr2: TGFβ type-II receptor, Vldlr: VLDL receptor, Zadh1: prostaglandin (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Up-regulation of sterol O-acyltransferase 1 (*Soat1*) increases cholesteryl ester (CE) level. Down-regulation of acetyl-CoA acyltransferase 1A (*Acaa1a*) and 2 (*Acaa2*) reduces fatty acid β-oxidation. Acyl-CoA synthetase bubblegum family member 1 (*Acsbg1*) help incorporating fatty acids into phospholipids.

Up-regulation of snail family transcriptional repressor 1 (Snai1), transforming growth factor beta 1 (*Tgfb1*), 2 (*Tgfb2*) and 3 (*Tgfb3*) as well as transforming growth factor beta receptor 2 (*Tgfbr2*) indicates the activation of TGF-β signaling pathway. Frizzled class receptor 1 (*Fzd1*) and 2 (*Fzd2*), transcription factor 4 (*Tcf4*), Kruppel like factor 4 (*Klf4*) are involved in the maintenance of cancer cell "stemness".

Prostate-specific Abca1 *deletion and* FASN *overexpression synergistically enhances the stemness of prostate cancer cells*

To corroborate the microarray data, we determined the expression of prostaglandin E receptor and TGF-β by qPCR and immunohistochemistry. qPCR results confirmed that the mRNA expression of *Ep3, Tgfb, Tgfbr2* was significantly increased in PAF compared to that in P (Fig. 6A). Immunohistochemistry data further supported the observation (Fig. 6B). In addition, the level of bicycle PGE$_2$, a stable form of PGE$_2$ metabolism, was elevated in PAF compared with the other three groups (Fig. 6C).

Next, we examined the stemness of tumor cells. Prostate cancer cells were stained with CD49f-PE, sca-1-APC and lineage specific antibodies (CD31-FITC, CD45-FITC and Ter119-FITC). Lin$^-$, sca-1$^+$ and CD49f high cells are considered as prostate cancer stem/progenitor cells (PCSC). Our results showed that the ratio of PCSC was 0.92%, 2.36%, 1.97% and 4.9% in P, PA, PF and PAF, respectively (Figure 6D). Spheroidizing ability is another characteristic of cancer cell stemness. P prostate cells were used in spheroidization experiment in the presence of palmitate, cholesterol alone or combination of both. Combination of palmitate and cholesterol significantly increased the number and size of spheres compared to the control, palmitate, cholesterol groups (Fig. 6E). Finally, tumorigenesis was evaluated *in vivo*. P or PAF cells were injected subcutaneously into nude mice and, after three weeks, tumors were harvested and weighed. Three fifths of P cell inoculums formed tumor, while 100% of PAF cells formed tumor. The tumors formed by PAF cells were also significantly larger (Fig. 6F).

Discussion

Prostate cancer is the seventh most common malignant male tumor, and its incidence and mortality are increasing. More importantly, prostate cancer is the only malignancy in men where both morbidity and mortality have increased significantly in the last 15 years in China (38). Therefore, it is urgent to explore the possible causes and seek effective prevention and treatment methods. Increasing evidence supports that saturated fatty acids and cholesterol are closely related to the occurrence of cancers. For example, studies have shown that saturated fatty acids in the diet have a positive correlation with the incidence of prostate cancer (10,39,40) and endogenous long-chain saturated fatty acid synthesis-related enzymes (ELOVL7) can promote the growth of prostate cancer (33). The expression of fatty acid synthase (FASN) is also upregulated in the early stage of prostate cancer (41,42). Elevated cholesterol levels were significantly associated with the development of prostate cancer, which is reduced by statins (43–45). However, there are also researchers who demonstrate that saturated fatty acids or cholesterol are not significantly related to the incidence of prostate cancer (17,46,47). Reason(s) for the inconsistency is (are) unclear. It may require both fatty acid and cholesterol, but not alone, to promote tumor proliferation. The present study demonstrates such a combinational effect of fatty acid and cholesterol on prostate cancer.

Experimental diets contain many other nutrients in addition to cholesterol and fat that may have confounding effects. Drugs like statins inhibit the

biosynthesis of several intermediate metabolites of cholesterol which are important for cellular functions, such as protein farnesylation and geranyl-geranylation (48,49). To overcome these potential shortcomings, mouse models with increased levels of cholesterol and fatty acids were generated by genetic approaches. We found that the combination of *FASN* overexpression and cholesterol *Abca1* knockout promotes prostate hyperplasia (Fig. 2), and converts Pten-null prostate HGPIN lesions to invasive PCa with 100% penetrance (Fig. 4). In addition, palmitate and cholesterol treatment increases prostate cell stemness (Fig. 6). These results suggest that although the incidence of asymptomatic and occult prostate cancer accompanied by genetic mutations is almost the same worldwide, the incidence of clinical prostate cancer will increase in countries where have high fat and cholesterol consumptions. This was and still is the case in the Western countries, and is becoming an issue in some Asian countries such as China.

With the improvement of living standards in China, dietary structure of residents is rapidly changing. The basic trend is a decrease in the consumption of cereals and potatoes and an increase in the intake of saturated fatty acids and cholesterol. From 1982 to 2002, the average daily fat consumption of Chinese has increased year by year, exceeding 30% of the recommended amount. Meanwhile, the proportion of saturated fatty acids in the total fatty acids has also increased from 26% to 29% (63, in Chinese). Recent studies have also shown that the percentage of the population eating more than the recommended amounts of fat and cholesterol has been rising, reaching 40.5 percent of adult men and 34.1 percent of adult women by 2009 (65,66, in Chinese). The U.S. Dietary Guidelines (2015–2020), which no longer limit the daily intake of cholesterol (previously no more than 300 mg/day), have a confounding effect on daily cholesterol intake. It is possible that cholesterol intake is not significantly related to the incidence of blood lipids and cardiovascular diseases. However, combinational effect of fatty acid and cholesterol on cancers warrants further investigation.

Altered lipid metabolism is increasingly recognized as a signature of cancer cells (50). The esterification of cholesterol is a biochemical process required for the storage of cholesterol in cells. Excessive cholesterol will cause cholesterol to exist in the lipid droplets inside cancer cells in the form of cholesteryl esters (51). Studies have shown that depletion of cholesteryl ester can significantly reduce the proliferation and invasion of prostate cancer cells. The Soat 1 inhibitor Avasimibe can inhibit the growth and metastasis of tumors by blocking the process of cholesterol esterification (52). Therefore, we suspect that excessive accumulation of cholesterol and fatty acids promotes the malignant transformation of prostate cancer via the formation of cholesteryl esters.

Our data indicate that PGE$_2$ and TGF-β pathways were activated in $P^{-/-}$; F^T; $A^{-/-}$ prostate tumors, and tumor stem/progenitor cell (PCSC) population increased significantly (Figs. 5 and 6). PGE$_2$ is an important inflammatory mediator produced by cyclooxygenase 2 (COX-2) metabolizing arachidonic acid (AA), and is considered to have strong pro-inflammatory and pro-tumor activities. PGE$_2$ receptors (EP1-4) are G protein-coupled receptors, and different subtypes are expressed in different cells. For example, breast epithelial stem cells express EP4 receptor, and PGE$_2$ can activate multiple signaling pathways including PI3K and Wnt after EP4 binding, and promote stem cell characteristics (53). The development and regeneration of hematopoietic stem cells are also related to the coordinated regulation of cAMP/PKA by PGE$_2$ and Wnt pathways (54). Studies have shown that the activation of the TGF-β pathway promote the proliferation of stem cell (55,56). Increased uptake of linoleic acid and synthesis of arachidonic acid can lead to higher levels of PGE$_2$ (Fig. 5). However, it is not clear how changes in lipid metabolism affect the TGF-β signaling pathway and whether PGE$_2$ and TGF-β synergistically enhance the stemness of prostate cancer cells.

In summary, the work presented here shows that fatty acid and cholesterol combination activates the PGE$_2$ and TGF-β pathways, enhances PCSC population. It provides a possible mechanistic explanation on why prostate
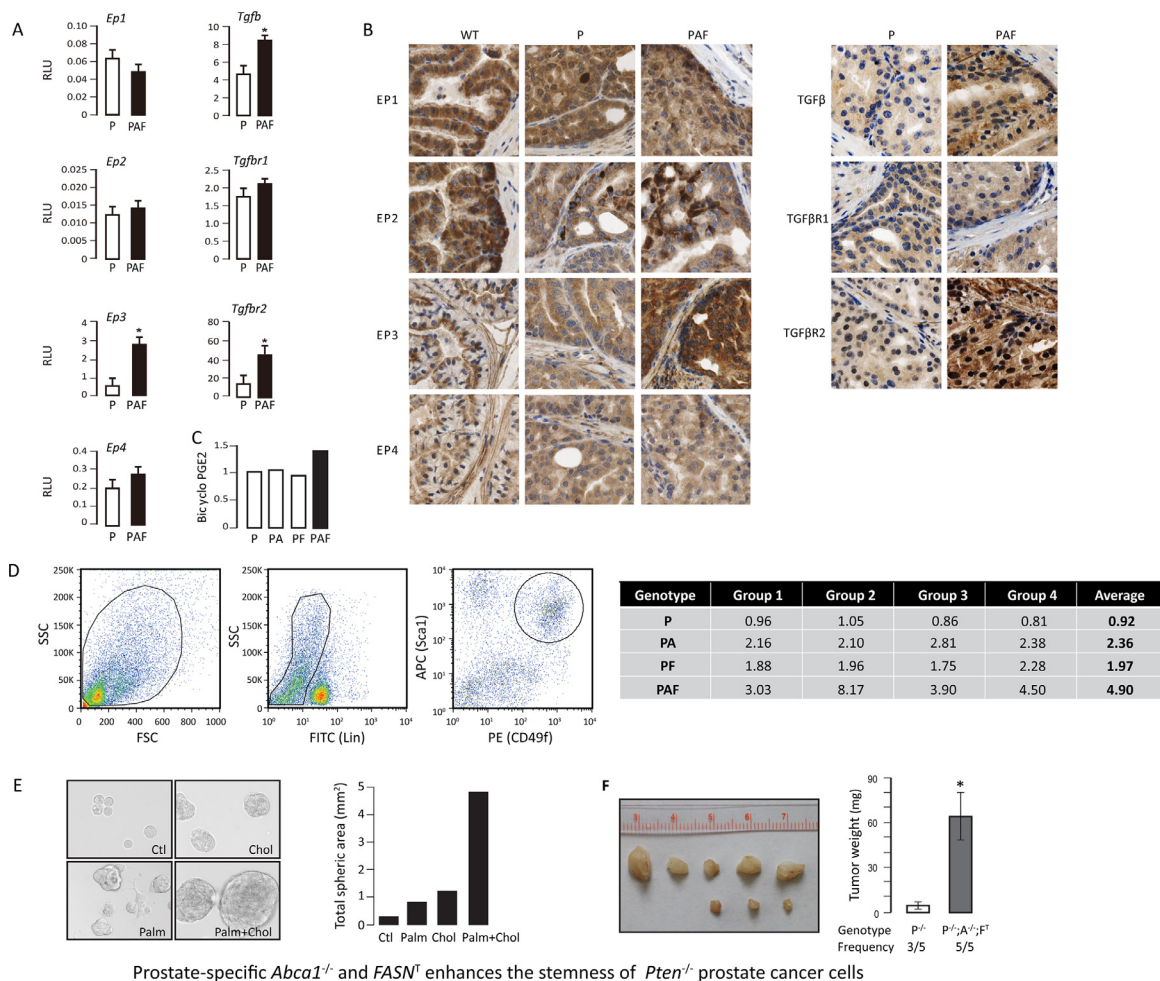
Fig. 6. Prostate-specific *Abca1* deletion and *FASN* overexpression synergistically enhances the stemness of prostate cancer cells

(A). qRT-PCR was performed to check the expression of EP1, EP2, EP3 EP4, TGFβ1, TGFβR1 and TGFβR2 (HPRT was used as reference). In $P^{-/-}$; F^T; $A^{-/-}$ tumor content of EP3, TGFβ1 and TGFβR2 mRNA was significantly upregulated. (B) . Immunohistochemical staining of PGE$_2$ receptors and TGFβ1/receptors. Prostate tissues from different genotypic mice were used. (Left): IHC results of EP staining. EP1 antibody was from Alpha Diagnostic International (San Antonio, TX). EP2, 3 and 4 antibodies were purchased from Cayman Chemical (Ann Arbor, Michigan). (Right): IHC staining of TGβ, TGFβRI and TGFβRII. Antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas). (C) . HPLC-MS was used to examine the production of bicycle PGE$_2$ in mice with different genotpyes. As shown, bicycle PGE$_2$ was increased in $P^{-/-}$; F^T; $A^{-/-}$ tumor. (D). The proportion of cancer stem cells in $P^{-/-}$; F^T; $A^{-/-}$ mice increased. (E). $P^{-/-}$; F^T; $A^{-/-}$ PCSC formed more and larger spheres than $P^{-/-}$ PCSC. (F). The tumors formed by $p^{-/-}$; F^T; $A^{-/-}$ PCSC were significantly larger than $P^{-/-}$ PCSC.

cancer morbidity and mortality are rising steadily in China in the last 15 years. Dietary intervention and/or Soat1 inhibition may be explored as new treatments for prostate cancer.

Materials and methods

Patient data: 1184 representative cases and 507 comparable controls from 2010 to 2017. The cases were selected from the patients with prostate cancer at the Cancer Registry of Wuxi CDC. According to the existing information, trace to the hospital where the patient visited, and inquire about the biochemical and pathological information of the patient at the first visit. The control is derived from an unbiased sample of the source population that produced the case. Based on the name and address information, the biochemical information of the control at the time of physical examination or hospitalization for other diseases is inquired. Exclude diseases such as urinary system, endocrine system and coronary heart disease. By querying the patient's laboratory test results which contain 58 biochemical indicators,

organizing the data and eliminating invalid indicators. The original data were all converted into categorical variables according to the fourth edition of Chinese clinical laboratory practice. A univariate analysis of all test results by chi-square test showed that in addition to the prostate cancer specific antigen (PSA) that can be used as a positive control, there are 8 indicators that are significantly different between patients and normal people, mainly lipid metabolism Related indicators. In order to further analyze the risk factors of prostate cancer, the indicators with statistical differences in the univariate test were selected for multivariate analysis to test the relationship between related factors and the incidence of prostate cancer.

Transgenic mice: Prostate-specific *Abca1*-knockout mice were generated by crossing *Abca1*$^{loxP/loxP}$ mice (57) with mice of the ARR2 probasin-*cre* transgenic line *PB-cre4*, wherein the Cre recombinase is under the control of a modified rat prostate-specific probasin promoter as previously reported (37). Prostatic FASN transgenic mice were generated using a construct that included the androgen-responsive probasin promoter (ARR2-PB), the full-length human FASN cDNA, an internal ribosome entry site, a luciferase

reporter gene, and a polyadenylation signal sequence (58). Prostate-specific Pten knockout mice were generated as described previously (59). The detailed protocols for genotyping and mouse breeding are presented in Supplemental Methods. These mice were then used to generate bi- and tri-transgenic mice by similar strategies as described as previously (59). The prostate tissues, liver and plasma collected from mice were snap-frozen in liquid nitrogen and stored at -80°C. Portions of prostate tissues were also formalin fixed, paraffin embedded and processed for H&E staining. Histopathological evaluation of mouse prostate tissues was performed by pathologists with extensive experience in murine pathology.

Fatty acid analysis: Fatty acid profiles were analyzed as described previously (59).

Cholesterol measurement: The cholesterol levels in prostate, liver and plasma were measured as described previously (60).

In vivo luciferase activity assay: The FASN-linked luciferase activity was determined in Xenogen IVIS 100 optical imaging system (Cliper Life Sciences, Hopkinton, MA). Each mouse was injected intraperitoneally with 100 μl of firefly luciferase substrate luciferin (3.5 mg/mL in PBS) and imaged up to 10 min in supine position. The mouse images were analyzed by the software provided with the imaging system.

Filipin staining of cholesterol: A filipin staining stock solution was prepared by dissolving 25 mg filipin (Sigma, St. Louis, MO) in 5 mL of DMSO and the light-protected solution was stored at –80°C until use. A working solution was prepared by adding 0.5 mL of stock solution into 49.5 mL of PBS. Frozen tissues were sectioned and fixed with freshly made 4% paraformaldehyde for 30 min at RT. After washing with PBS, sections were then incubated with the working solution for 30 min at RT in the dark. Slides were mounted with a cover slip using Prolong Gold anti-fade reagent (Invitrogen, Grand Island, NY) after rinsing with PBS. Cholesterol staining was examined under a fluorescent microscope with excitation of 365 nm and emission of 405 nm wavelength.

ABCA1 knockdown in vitro: Human prostate cancer cells C4-2 were infected with lentivirus expressing the GFP tag and *ABCA1* targeting shRNA (GGA CCT GAC AGG AAG AAA CAT T) or control shRNA (GGG CCA TGG CAC GTA CGG CAA G). Infected cells were purified by flow cytometric sorting of GFP positive cells.

FASN virus infection: Briefly, 293T cells were transfected with either an empty pSL4 vector or pSL4-FASN together with three packaging plasmids (pMDLg/pRRE, pRSV-RSE and pVSV-G) using a transfection protocol of calcium phosphate-DNA precipitation. Medium containing viral particles was collected 48 h after transfection. Lentiviruses were added to the cells in a medium containing 8 μg/mL polybrene (61). The FASN virus infected cells were selected with 2 µg/mL of puromycin and validated by Western blotting of FASN.

Real-time PCR: Total RNA was extracted using TRIZOL (Invitrogen) and reverse transcribed with Superscript III plus RNase H-Reverse Transcriptase (Invitrogen). Real-time polymerase chain reaction was performed with Platinum SYBR Green qPCR Supermix UDG (Invitrogen) using a Bio-Rad iCycler (Hercules, CA) as described (61). The primers used were forward primer AAA TGG TGA AGG TCG GTG TG and reverse primer CGT TGA ATT TGC CGT GAG for *Gapdh*.

Western blot: Cells were treated with a combination of the 2 compounds: 10 μmol/L T0901317 (Sigma) and 5 μmol/L 5-aza-2-deoxycytidine (Sigma) for 5 days (62). For Western blot analysis of ABCA1 and β-actin, cultured cells were lysed in RIPA buffer composed of 50mM Tris-HCl, 150mM NaCl, 0.1%SDS, 1% Sodium deoxycholate, 1mM EDTA, 1mM PMSF, and cocktail protease inhibitor (Roche). Equal amounts of protein lysates (about 50 μg/lane) were separated by Bis- Tris gel, transferred onto nitrocellulose membranes, which were blocked in 3% milk for 1 h at RT and blotted with antibody overnight at 4°C: anti- ABCA1 (ab66217, AbCam; 1:1000). Peroxidase-conjugated 2° antibody (ECL) was used at 1:3,000 for 1hr at RT.

Statistics: Quantitative data are presented as mean $\pm$ SD. Data were analyzed by ANOVA with Tukey post-hoc tests, unpaired Student's t-test, or χ^2 test (Prism 5 GraphPad Software) as noted in the figure legends, with $p < 0.05$ considered as statistically significant. Spss22.0 software was used to establish a database for analysis, and the comparison of categorical variables was described by composition ratio. Single-factor logistic regression analysis was used, and the forward method was used to perform multi-factor logistic regression analysis on meaningful variables ($P \leq 0.05$ is considered statistically significant).

Ethics approval

Pca tissues were collected from patients who underwent surgical resection at the Affiliated Hospital of Jiangnan University (Wuxi, China). All experiments were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of the Jiangnan University (JNU. No201701IRB2). All animal procedures were performed accordance with the Guidelines for Care and Use of Laboratory Animals of Research Institute of Schistosomiasis Control in Jiangsu Province and experiments were approved by the Animal Ethics Committee of Research Institute of Schistosomiasis Control in Jiangsu Province, China (JN. No20180915b04011020).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2021.11.004.

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